The Role of VEGF in Macrophage Recruitment During Wound Healing

Undergraduate Honors Thesis

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ABSTRACT

Inflammation is an important first step in the wound healing process. During inflammation, macrophages are recruited to the wound site to clear debris, kill microbes and mediate subsequent steps of repair. Vascular endothelial growth factor (VEGF) is produced at high levels in wounds, and it is best known for stimulating new blood vessel growth by binding to VEGF receptors on endothelial cells. However, VEGF receptor-1 (VEGFR-1) is also expressed on the surface of macrophages and VEGF has been shown to induce migration of macrophages *in vitro*. We hypothesize that VEGFR-1 is important in the migration of macrophages to a wound site in response to VEGF. We generated a unique conditional knockout mouse strain that lacks VEGFR-1 only in macrophages using the Cre-lox system. These mice were utilized to explore the role of VEGF in macrophage recruitment to wounds *in vivo*. Wound healing studies were performed in conditional macrophage VEGFR-1 knockout mice (KO) and control mice. Full thickness excisional wounds were administered on the dorsal skin using a 3mm biopsy punch. Wounds were collected after 5 days for analysis of macrophage recruitment and wound closure. First, macrophage recruitment was quantified in wounds by immunohistochemistry. Wound sections were stained for F4/80, a macrophage specific cell surface marker, and the number of macrophages was counted. Control wounds contained a significantly higher average density of macrophages when compared to knockout wounds. Wound closure was also assessed by measuring reepithelialization in H&E stained wounds. Only 15% of knockout wounds exhibited complete wound closure, compared to 42% in control wounds, suggesting that wounds in knockout mice healed more slowly. Angiogenesis and cell proliferation were also assessed, but no differences were observed between knockout and control groups. These

studies point to a direct role of VEGF:VEGFR-1 signaling in the recruitment of macrophages to a wound site and suggest that a reduction in macrophages may lead to delayed wound healing. Defining the pathway by which macrophages are recruited to a wound will broaden our understanding of the wound healing process. This could lead to better therapeutic methods in the treatment of wounds to increase healing efficiency and reduce abnormal wound healing.

Chapter 1:

INTRODUCTION

1.1 Wound Healing: The skin is the largest organ of the body and serves as a barrier between the body and the outer environment. It consists of two layers, the epidermis and the underlying dermis. When an injury is inflicted on the skin, a wound healing process begins. Wound healing consists of three overlapping phases: inflammation, proliferation, and scar formation (Singer, 1999; Gurtner, 2008).

The first of these phases, inflammation, occurs immediately following injury, and is characterized by an influx of immune cells in response to various chemotactic signals. In this phase, neutrophils, a type of immune cell, are attracted to the wound to help clear debris and kill bacteria (Martin, 1997). Additionally, local resident macrophages near the wound are activated, causing them to recruit immature monocytes from the blood. The monocytes then differentiate into mature macrophages in the wound (Koh, 2011). Upon recruitment to the wound site, macrophages engulf bacteria, clear debris and dead cells, and mediate a number of processes during the next phase of proliferation (Delavary, 2011).

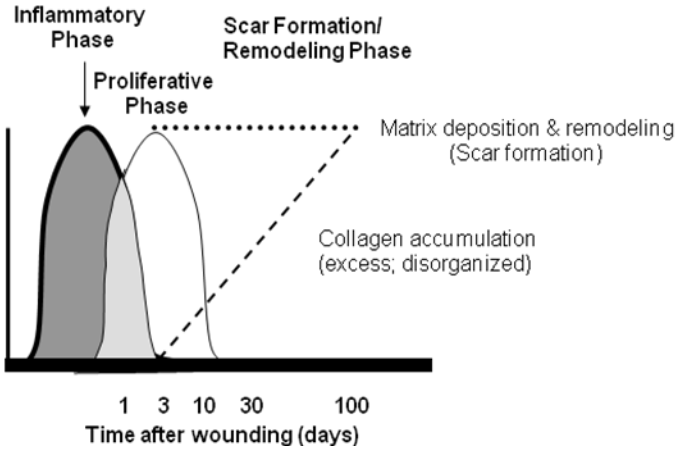

Figure 1: The Phases of Wound Healing. Wound healing occurs in three overlapping phases: inflammation, proliferation and scar formation. Adapted from Clark, 1996.

During the proliferative phase, keratinocytes, a cell type in the epidermis, proliferate and migrate over the wound bed to reform the epidermal layer of the skin (Martin, 1997). New

blood vessels are also formed during the proliferative phase through angiogenesis. Fibroblasts also begin proliferating and migrating toward the wound bed during this phase (Singer, 1999).

The last stage of wound healing, scar formation, occurs as a slow process, during which fibroblasts produce collagen to form a new matrix within the wound site (Martin, 1997). The collagen is then remodeled by fibroblasts, forming a permanent scar (Martin, 1997).

1.2 Angiogenesis: Angiogenesis, the development of new blood vessels from pre-existing vessels, is required for embryonic development and also occurs during normal wound healing (Dvorak, 2005). Angiogenesis plays a crucial role in the repair process and occurs during the proliferative phase of healing. It is through the new blood vessels formed during angiogenesis that nutrients and oxygen are delivered to the wound site, enabling the healing process to proceed (Martin, 1997). One of the most important regulators of angiogenesis is a protein known as vascular endothelial growth factor (VEGF) (Nissen, 1998).

1.3 VEGF/VEGFR-1: Vascular endothelial growth factor (VEGF) is a protein found in great amounts in the wound bed (Nissen, 1998). VEGF is produced by keratinocytes and infiltrating immune cells, like macrophages, in response to injury (Brown, 1992; Nissen, 1998; Willenborg, 2012). VEGF promotes angiogenic signaling by interacting with one of several tyrosine kinase receptors on endothelial cells including VEGFR-1 (flt-1) and VEGFR-2 (flk-1) (Ferrara, 2003). VEGF signaling in endothelial cells results in a number of cellular responses including vascular

permeability, cell survival, and cell proliferation (Dvorak, 2005). Recently, VEGFR-1 has also been shown to be expressed on non-endothelial cells such as keratinocytes (Man, 2006; Wilgus, 2005) and macrophages (Sawano, 2001; Barleon, 1996), suggesting other roles of VEGF beyond angiogenesis. Several studies have determined that VEGFR-1 is expressed on macrophages and that migration of macrophages grown in cell culture is stimulated by VEGF (Sawano, 2001; Barleon, 1996).

1.4 Macrophages: Macrophages, an innate immune cell, have been shown to be critical in orchestrating several steps of the wound healing process. Two populations of macrophages are found in the skin following injury. The first of these are resident macrophages, which are located normally in unwounded dermis. The second population of macrophages is recruited during wound healing and begins infiltrating the wound site during the inflammation stage. During this recruitment process, circulating monocytes (immature macrophages) leave the vasculature, enter the wound bed, and mature into macrophages where they help to mediate the wound healing process (Gurtner, 2008). The number of macrophages peaks between 3-5 days after the initial injury (Koh, 2011). The activation of macrophages within a wound is thought to be critical for proper wound repair, because depletion of macrophages results in delayed wound healing (Leibovich, 1975; Mirza, 2009; Lucas, 2010).

Wound macrophages are typically divided into two phenotypes: M1 (classically activated) or M2 (alternatively activated) (Novak, 2013). M1 macrophages, activated by bacterial products or inflammatory cytokines, are found early in wound healing and help to

facilitate antimicrobial responses and mediate initial inflammatory processes by secreting tumor necrosis factor (TNF)-α , nitric oxide (NO), and interleukin (IL)-6 (Koh, 2011). M2 macrophages, a more heterogeneous population found later in the wound healing process, are activated by a number of interleukins (IL-4, IL-13) and help mediate the proliferative and remodeling processes within the wound bed (Koh, 2011). An imbalance of one of the phenotypes could be detrimental, as seen in chronic wounds. Chronic wounds contain more pro-inflammatory M1 macrophages and less pro-resolution M2 macrophages, which is thought to contribute to persistent inflammation and cause delayed healing (Khanna, 2010; Novak, 2013).

The role of the macrophages within the wound healing process has been studied extensively, but the role of VEGF in macrophage recruitment and macrophage function is not well characterized. Due to the importance of macrophages in wound healing (Leibovich, 1975; Mirza, 2009; Lucas, 2010; Koh, 2011; Devalary, 2011), a better understanding of what controls macrophage migration important. The goal of this project is to determine whether VEGF is involved in macrophage migration within a wound setting.

1.5 Rationale and Hypothesis: From data produced in previous studies, it is known that VEGF is produced in wounds, VEGFR-1 is expressed on the cell surface of macrophages, and VEGF induces macrophage migration via VEGFR-1 *in vitro*. Therefore, our goal was to determine whether VEGF mediates macrophage migration *in vivo*. *We hypothesized that VEGF:VEGFR-1 signaling is important in the migration of macrophages to a skin wound.*

To test this hypothesis, we created a unique conditional knockout mouse containing macrophages that do not express VEGFR-1. Therefore, the macrophages in these mice cannot to respond to VEGF since they do not have the VEGFR-1 receptor. Macrophage numbers were compared in wounds from knockout mice and control mice to determine whether the absence of VEGFR-1 has a direct effect on macrophage recruitment. Macrophage numbers at the wound site could affect wound healing; therefore, wound closure was also compared in knockout mice and control mice.

Overall, we observed decreased macrophage numbers in wounds from the conditional knockout mice when compared to control wounds. We also saw slower healing in knockout mice compared to wounds from control mice, suggesting an indirect role of macrophages in the reepithelialization of wounds as a result of VEGF:VEGFR-1 inhibition.

Chapter 2:

METHODS

2.1 Mouse Model: A unique conditional knockout mouse strain was created that exhibits an ablation of the VEGFR-1 gene in macrophages, utilizing the Cre-Lox system. VEGFR-$1^{fl/fl}$ mice contain two copies of a VEGFR-1 gene in which exon 1 is flanked by two Lox P sites. VEGFR-$1^{fl/fl}$ mice were bred with mice expressing Cre recombinase under the control of the monocyte/macrophage specific LysM (lysozyme M) promoter (Claussen, 1999) **(Fig 2A)**. Expression of Cre recombinase deletes the portion of VEGFR-1 gene that is flanked by the Lox P sites, leaving one loxP site **(Fig 2B)**. The result is a LysMCre/VEGFR-$^{1fl/fl}$ conditional knockout (KO) strain that lacks expression of the VEGFR-1 gene only in macrophages.

Genotypes were confirmed by isolating DNA from tail snips of pups using the DNeasy kit (Qiagen). Mice were genotyped by PCR (polymerase chain reaction) to detect the presence of the floxed VEGFR-1 gene using specific primers that flank the second loxP site, generating a 219

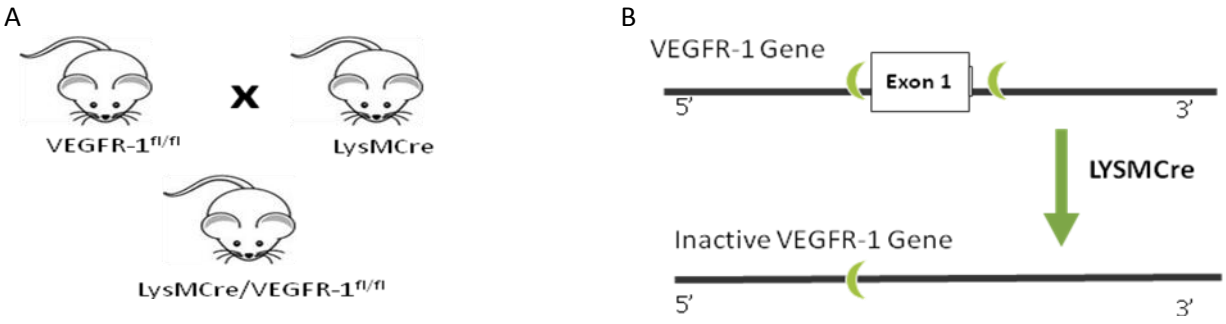

Figure 2: Conditional Knockout Mouse Model. Mice containing a VEGFR-1 gene with exon 1 flanked by LoxP sites (VEGFR-$1^{fl/fl}$) were bred with mice expressing Cre recombinase under the control of the macrophage specific LysM promoter (LysMCre), yielding a unique conditional knockout mouse (LysMCre/VEGFR-$1^{fl/fl}$) (A). When Cre is expressed in macrophages, it deletes the portion of the VEGFR-1 gene located within the two Lox P sites, resulting in deletion of exon 1 (B).

basepair (bp) band in control mice or a 396 bp band in floxed mice **(Figure 3)**. Two primer sets were used to detect the presence of the wild type lysosome M gene or the LysMCre transgene **(Figure 3)**. PCR products were visualized via gel electrophoresis. Briefly, PCR products were loaded on a 2.5% agarose gel which was run at 100V for 40 minutes. The gels were then stained with ethidium bromide (0.625 µg/ml). Bands were visualized with an ultraviolet light transilluminator and digital images were taken using a Chemidoc imaging system (Bio-Rad).

2.2 Magnetic Cell Sorting and Flow Cytometry: Macrophages were isolated from the spleens of knockout and control mice using MACS magnetic cell sorting. A single cell suspension of splenocytes was incubated with magnetic beads coupled to an antibody for CD11b, a macrophage marker. $CD11b^+$ cells were separated from $CD11b^-$ cells via exposure to a magnetic field (Miltenyi). $CD11b^+$ cells from knockout and control mice were stained with a PE (phycoerythrin)-conjugated VEGFR-1 antibody (R&D Systems) and analyzed by flow cytometry.

2.3 Macrophage Cell Culture and Gene Expression Analysis: To confirm functional loss of VEGFR-1, bone marrow derived macrophages (BMDM) were cultured from bone marrow isolated from femurs of knockout and control mice. Bone marrow cells were washed 2 times by centrifugation, counted and plated at $4x10^6$ cells per well into 6-well tissue-culture treated plates. BMDM were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS), 1% HEPES, 1% penicillin-streptomycin (all from Invitrogen), and 20% L929-cell conditioned media (as a source of monocyte-colony stimulating factor to drive macrophage maturation). Twenty-four hours following plating, wells were washed twice with

PBS (phosphate buffered saline) to remove all non-adherent cells and growth media was replaced. Media was changed every other day thereafter.

After 7 days, total RNA was isolated from BMDMs using TRIzol reagent (Invitrogen Corp., Carlsbad, CA). Briefly, media was removed and 0.5 ml of TRIzol was added to each well. Plates were rocked on ice for 5 minutes, then wells were scraped with a pipet tip and the TRIzol was transferred to a 1.7ml microcentrifuge tube. 0.2 ml of chloroform was added to each tube. Tubes were shaken vigorously for 15 seconds and incubated for 2 minutes at room temperature. Following centrifugation at 12,000xg at 4^0C for 15 minutes, the upper aqueous layer was transferred to a new tube and 0.5 ml of isopropanol was added. Following a 10 minute incubation at room temperature, tubes were centrifuged for 10 minutes at 12,000xg and 4^0C. The supernatant was removed and the pellet washed with 1 ml of 75% ethanol and centrifuged at 7,500xg for 5 minutes at 4^0C. The supernatant was removed and the pellets were air dried for 15-20 minutes. The RNA pellet was resuspended in 25 µl of RNase/DNase free water and heated for 10 minutes at 60^0C.

RT-PCR (Reverse transcriptase-polymerase chain reaction) was performed on RNA isolated from BMDMs using the following primers: mouse VEGFR-1 sense 5' ATA AGG CAG CGG ATT GAC 3', mouse VEGFR-1 anti-sense 5' CCA GCG GAT AGA GAG GTG 3', β-actin sense 5' CCC TGG AGA AGA GCT ATG AG 3', β-actin anti-sense 5' GGC ATA GAG GTC TTT ACG GA 3'. Briefly, 1 µg of total RNA was reverse transcribed at 42°C for 15 minutes in 20 µl containing 12.5 mM Tris HCl, 18.75 mM KCl, 0.75 mM MgCl, 5 mM DTT, 0.5 mM dNTP mix, 0.5 µg oligo(dT) primers, 2 U M-MLV reverse transcriptase (all from Invitrogen Corp., Carlsbad, CA). 2 µl of the RT reaction was used for PCR in a reaction containing 12.5 µl of ReadyMix (Sigma), 1 µl of each primer and

8.5 µl of water. PCR reactions were heated at 95^0C for 10 minutes after which 30 cycles of PCR was performed (denature at 94^0C for 30 seconds, anneal at 60^0C for 30 seconds and elongate at 72^0C for 60 seconds). PCR products were run on a 2% agarose gel at 100V. Gels were stained in 0.625 µg/ml ethidium bromine for 30 minutes then destained in water for 30 minutes. Bands were detected using the ChemiDoc imaging system (Bio Rad).

2.4 Wounding: Female LysMCre/VEGFR-$1^{fl/fl}$ knockout mice and age matched control mice were wounded at 8 weeks of age. Mice were shaved along the dorsal skin and a series of three pairs of excisional wounds were administered using a 3mm biopsy punch generating a total of 6 wounds per mouse. Wounds were allowed to heal for 5 days, at which time they were sacrificed. Wounds were collected, embedded in tissue freezing media (Triangle Biomedical) and frozen.

2.5 Immunohistochemical Staining: Immunohistochemistry was used to identify macrophages, blood vessels, and proliferating cells. Frozen samples were sectioned (10 µm thickness) using a cryostat. Slides were thawed and fixed in acetone. After, sections were washed in PBS and subjected to a methanol and hydrogen peroxide block. Sections were again washed and blocked with 10% serum for 30 minutes, then incubated with primary antibody overnight at 4°C in a humidified chamber. Primary anti-F4/80 antibodies were used to detect macrophages, anti-PECAM-1 antibodies were used to detect endothelial cells in blood vessels, and anti-Ki-67 antibodies were used to detect proliferating cells. Sections were washed in PBS and incubated

with an appropriate biotinylated secondary antibody for 30 minutes. After washing in PBS, sections were incubated in peroxidase-labeled avidin-biotin complex (Vector Laboratories) for 30 minutes, then 3, 3'-diaminobenzidine (DAB) solution (Kirkegaard and Perry Laboratories) for 10 minutes in the dark. Finally, samples were counterstained with hematoxylin-2, dehydrated in graded ethanols, then cleared in Clear-rite (Richard-Allan Scientific). Slides were cover-slipped using Permount (Fisher Scientific).

2.6 Hematoxylin and Eosin Staining: Frozen samples were cut in 10 µm sections using a cryostat. Slides were thawed and stained with hematoxylin and eosin (H&E) (both from Richard-Allan Scientific) using standard procedures.

2.7 Image Analysis: Images of stained wounds were used to calculate cell densities and wound closure measurements. Macrophages were manually counted in the wound margins and Axiovision software (Zeiss) was used to determine the total area analyzed. The density of macrophages was determined by dividing the number of $F4/80^+$ cells per area in mm^2. Reepithelialization was calculated in H&E stained sections. Percent reepithelialization was calculated by dividing the distance that the new epithelial layer had migrated across the wound by the total wound bed width, then multiplying by 100. For PECAM-1 stained vessels and Ki-67 stained proliferating cells, Image J software (NIH) was used to outline the dermal wound bed and calculate the density of positive staining (percent positive area).

2.8 Statistics: Statistical analysis of data was performed using a Student's t-test. P-values of less than 0.05 were considered significant.

Chapter 3:

RESULTS

3.1 Validation of Conditional Knockout Mouse Model: Before experiments could be carried out, the LysMCre/VEGFR-1$^{fl/fl}$ needed to be created and confirmed as an effective model of VEGFR-1 ablation in murine macrophages. Initial genotyping was conducted using DNA isolated from tail snips of 2-3 week old mice. Mice containing at least one copy of the LysMCre allele and two floxed VEGFR-1 alleles (LysMCre/VEGFr-1$^{fl/fl}$) were considered knockout mice **(Figure 3)**.

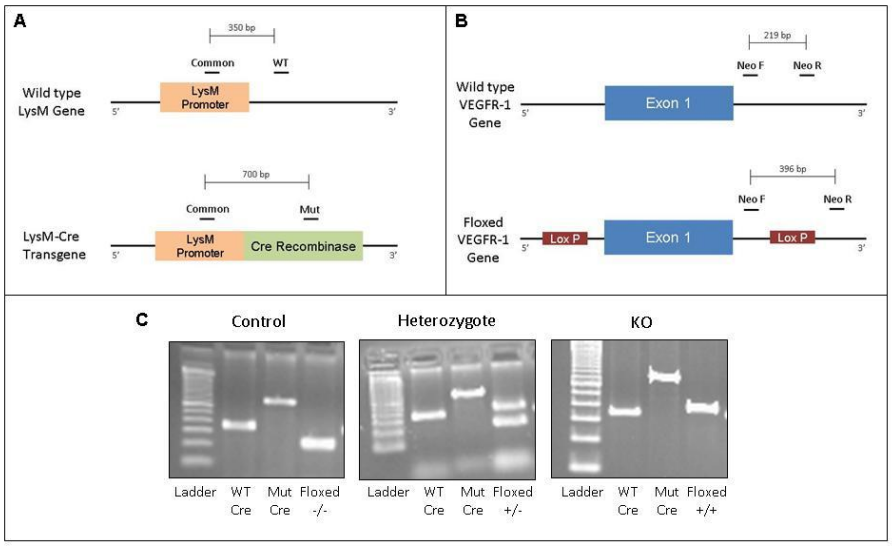

Figure 3: Mouse genotyping. The schematics in the top panels show the strategy used for genotyping. Two PCR reactions were used to detect the presence of the wild type LysM gene or the LysMCre transgene (A). For both reactions, a common primer was used that binds to the LysM promoter, which is present in all mice. For the first reaction, the common primer is used with a primer that recognizes a DNA sequence downstream of the LysM promoter (WT primer). For the second reaction, the common primer is used with a primer that binds a sequence within the inserted Cre recombinase gene (Mut primer). A single PCR reaction was used to determine whether mice contained floxed VEGFR-1 alleles (B). The Neo F and Neo R primers bind to two portions of the VEGFR-1 gene that surround the second LoxP site such that the PCR products differ in size depending on whether a wild type or floxed allele is present. Representative agarose gels for control, heterozygous and knockout (KO) VEGFR-1 mice are shown in C. For each gel, a 100 bp ladder is shown in the far left lane, followed by PCR reactions using the common and WT primers, common and Mut primers, and Neo primers. All mice are heterozygous for the LysMCre transgene, indicated by the presence of a band for wild type LysM and LysMCre. Control mice have do not have a floxed allele (single 219 bp band), heterozygous mice have one wild type and one floxed allele (bands at 219 bp and 396 bp), and knockout mice have two floxed alleles (single 396 bp band).

To confirm effective recombination in knockout mice, DNA was isolated from splenocytes which contain a large number of macrophages. As shown in **Figure 4**, PCR was used to confirm that exon 1 of the VEGFR-1 gene was deleted due to expression of Cre recombinase in knockout mice.

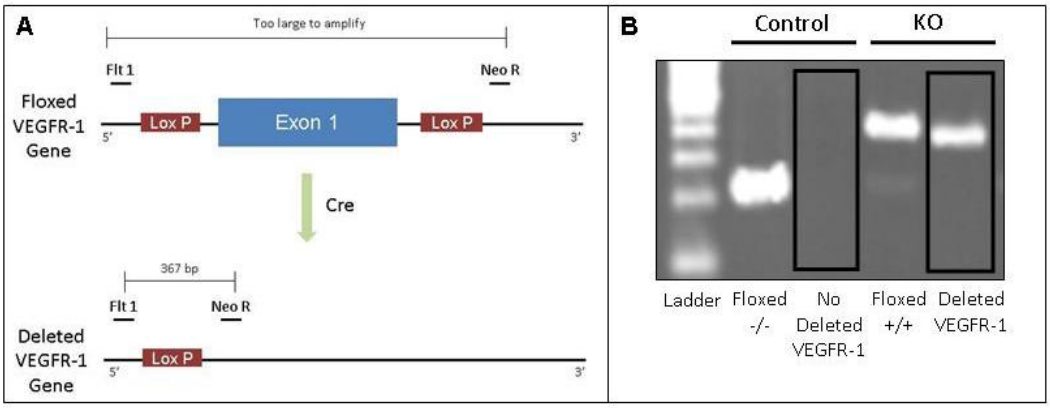

Figure 4: Detection of VEGFR-1 exon 1 deletion. The schematic in A shows the strategy used for detecting deletion of exon 1 of the VEGFR-1 gene (B). The PCR reaction uses flt-1 and Neo R primers, which bind to areas in VEGFR-1 gene that surround exon 1 and the LoxP sites. In mice that lack Cre recombinase, exon 1 will still be present and the predicted PCR product is too large to be amplified. In cells that express Cre recombinase, exon 1 will be deleted, leaving one LoxP site, and a 367 bp product is amplified. DNA was isolated from splenocytes of knockout and control mice (B). Exon 1 deletion was confirmed by PCR using the strategy described in A (in lanes highlighted with boxes). The presence of a 367 bp band in knockout mice shows deletion of exon 1. No band is amplified in control mice. Genotyping results for the floxed VEGFR-1 allele are also shown for each sample as a reference.

To confirm that VEGFR-1 expression was reduced on the surface of macrophages from knockout mice, $CD11b^+$ cells were enriched from knockout and control splenocytes using magnetic beads and subjected to flow cytometry. As expected, **Figure 5** shows a reduced number of macrophages expressing VEGFR-1 in knockout mice. The small number of $VEGFR\text{-}1^+$ cells detected in knockout mice could be attributed to nonspecific binding of cells to the CD11b antibody during separation. These results suggest that the conditional knockout model is functional.

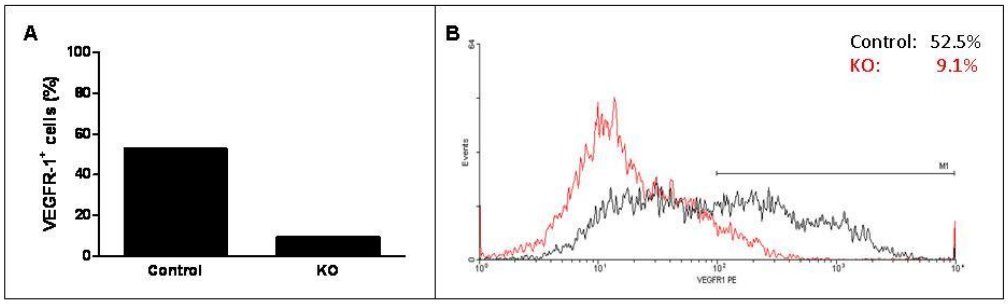

Figure 5: **Detection of VEGFR-1 protein in macrophages from control and knockout mice.** $CD11b^+$ macrophages were enriched from spleens of control and knockout mice using magnetic cell sorting. Flow cytometry was used to determine the percentage of cells expressing VEGFR-1 on the cell surface. The percentage of VEGFR-1^+ cells in control and knockout mice is shown graphically in A and a histogram of the data is shown in B. In control mice, 52.5% of cells expressed VEGFR-1 compared to 9.1% in knockout mice.

To confirm a reduction in VEGFR-1 expression in a more pure cell population, RNA was isolated from cultured bone marrow derived macrophages (BMDMs) from knockout and control mice. RT-PCR was used to analyze VEGFR-1 gene expression. VEGFR-1 mRNA nearly disappears in BMDMs from knockout mice, while VEGFR-1 expression was detected in cells from control mice **(Figure 6)**.

Overall, we determined that the LysMCre/VEGFR-$1^{fl/fl}$ knockout strain developed in our laboratory is an effective model of VEGFR-1 ablation in macrophages.

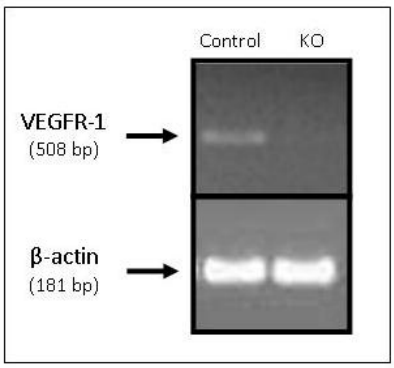

Figure 6: VEGFR-1 gene expression in cultured BMDMs from control and knockout mice. RNA was isolated from BMDMs and RT-PCR was used to determine VEGFR-1 mRNA levels (top panel). Detection of a 508 bp band indicates the presence of VEGFR-1 mRNA in BMDMs from control mice (left lane). No band is visible in BMDMs from knockout mice (right lane), indicating reduced VEGFR-1 expression. Expression of the housekeeping gene β-actin is also shown for each sample (bottom panel).

3.2 Macrophage quantification

To determine whether inhibition of the VEGF:VEGFR-1 pathway would affect macrophage recruitment *in vivo*, dermal wounds were administered in knockout and control mice. A time point of 5 days was chosen for analysis because macrophage numbers peak in a wound between 3-5 days after wounding (Delavary, 2011). Immunohistochemical staining for the macrophage specific cell marker F4/80 yielded visibly stained macrophages that could be quantified manually. A significantly lower number of macrophages were observed in the wound bed in wounds from knockout mice (355 cells/mm^2) compared to control wounds (497 cells/mm^2) **(Figure 7)**. These results suggest that the ability of macrophages to respond to VEGF through VEGFR-1 is important for macrophage migration to a wound.

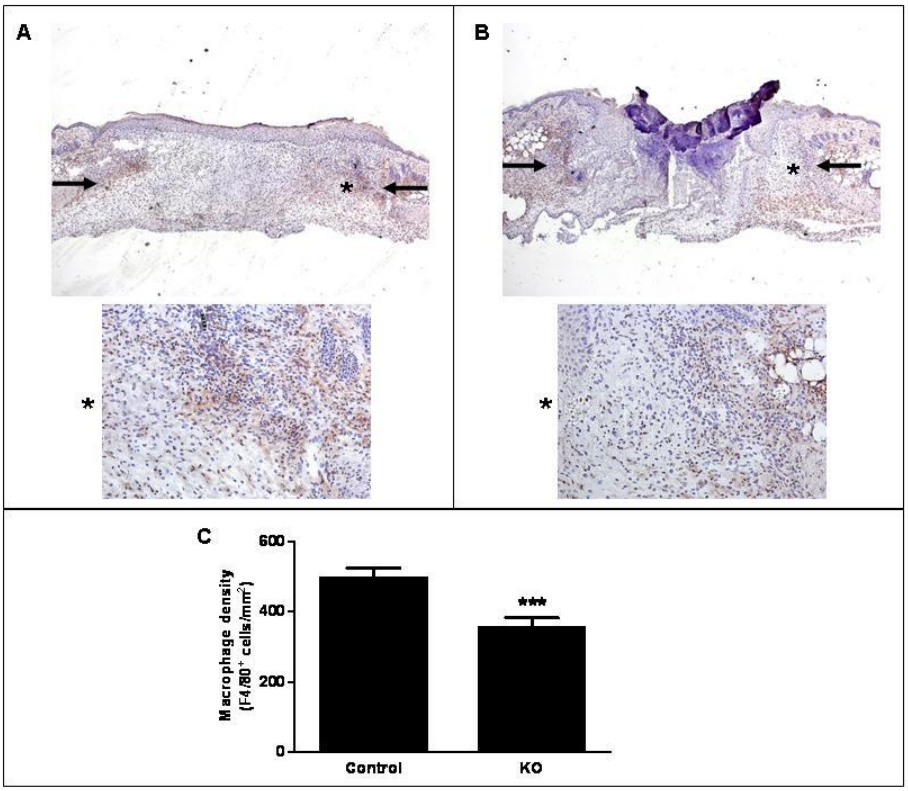

Figure 7: Quantification of macrophages in control and knockout wounds. Immunohistochemistry was used to detect F4/80-positive macrophages in 5 day wounds. Sample sections from wounds taken from a control mouse (A) and knockout mouse (B) are shown. In the top images, the margins of the wound bed are marked by arrows. The bottom images show a higher magnification of the area marked with an asterisk (*). Macrophages were quantified in each side of the wound edge and the area was determined using an image analysis program. The density of positive cells is shown in C. Bars represent mean macrophage density +/- S.E.M.; ***p=0.0008; n=19 control wound and n=15 knockout wounds.

3.3 Measurement of wound reepithelialization

Macrophages secrete cytokines that mediate keratinocyte proliferation and migration in the epidermis, which is important for wound closure or reepithelialization. Therefore, wound closure was also analyzed in knockout and control mice **(Figure 8)**. Noticeable differences in healing were observed between the two populations of mice. It is evident in the H&E-stained sections shown that knockout wounds **(Figure 8b)** appeared less healed than control wounds **(Figure 8a)**, with little keratinocyte coverage and extensive scabbing. The underlying dermal matrix also appears much more disorganized in knockout wounds, suggesting a delayed healing process. Surprisingly, when percent reepithelialization was determined (the percentage of the wound bed covered by keratinocytes) no statistical differences were observed between knockout (67%) and control wounds (80%) **(Figure 8c)**. However, a lower incidence of complete wound closure (wounds showing 100% reepithelialization) was seen in knockout wounds (15%) compared to control wounds (42%) **(Figure 8d)**. Overall, the data suggest that wound healing is delayed in knockout mice.

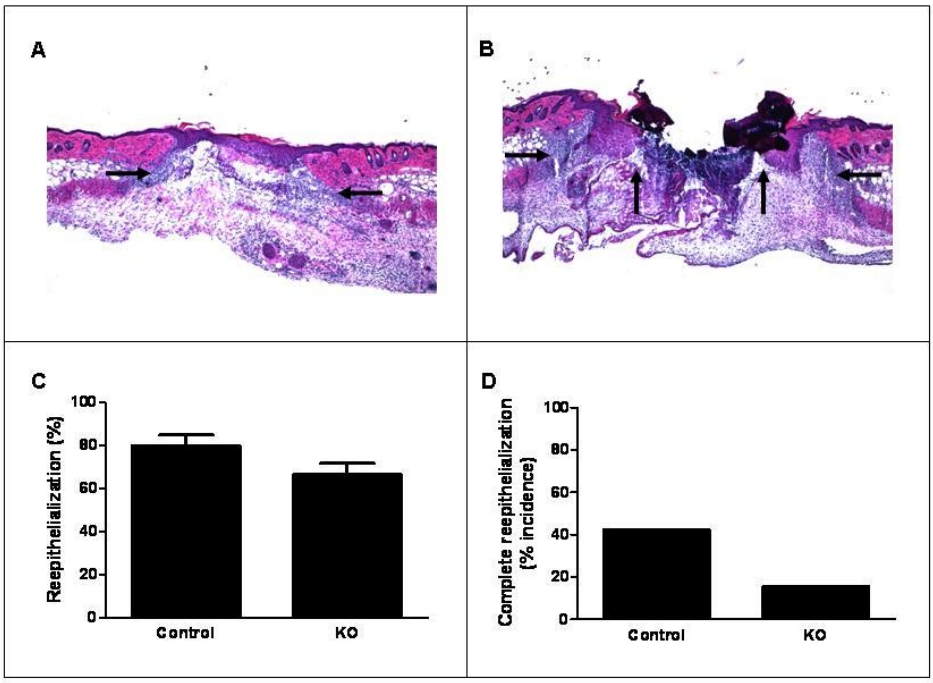

Figure 8: Reepithelialization of control and knockout wounds. Wound closure was assessed histologically in 5 day H&E stained wounds. Representative images of wounds from a control mouse (A) and a knockout mouse (B) are shown. Horizontal arrows indicate the edges of the wound bed and vertical arrows show the epithelial edges. The percentage of the wound bed covered by new epithelium (% reepithelialization) is shown in C. Bars represent mean percent reepithelialization +/- S.E.M.; p=0.0862. The percentage of wounds displaying 100% reepithelialization (complete epidermal closure) is shown in D. For both graphs, n=19 control wound and n=13 knockout wounds.

3.4 Analysis of Wound Angiogenesis

Because macrophages secrete a number of mediators that facilitate the formation of new blood vessels during the proliferative phase of wound healing, wound angiogenesis was compared in knockout and control mice. Immunohistochemical staining for PECAM-1, an endothelial cell marker, was used to characterize blood vessel density in knockout and control wounds **(Figure 9)**. Image analysis software data, shown graphically in **Figure 9c,** indicated that there was no statistical difference in angiogenesis between knockout and control wounds.

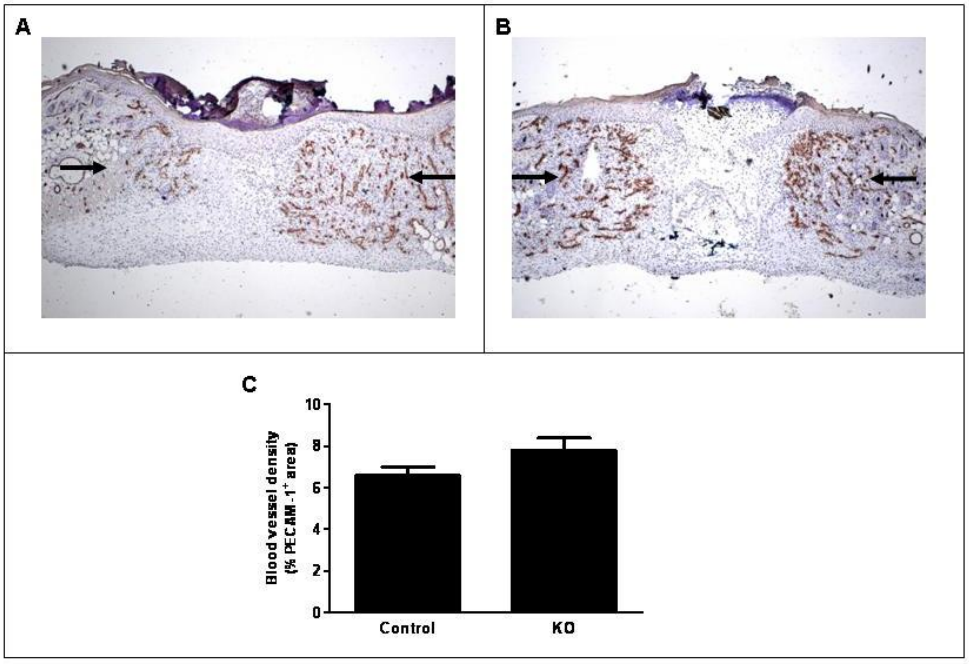

Figure 9: Analysis of angiogenesis in control and knockout wounds. Immunohistochemical staining for the endothelial cell marker PECAM-1 was used to detect blood vessels in 5 day wounds. Sample sections from wounds taken from a control mouse (A) and knockout mouse (B) are shown. The margins of the wound bed are marked by arrows. Stained sections were analyzed using image analysis software to determine blood vessel density, or the percent area of the total wound bed stained positive for PECAM-1 (C). Bars represent mean blood vessel density +/- S.E.M.; p=0.1058; n=19 control wound and n=16 knockout wounds.

3.5 Analysis of Proliferation:

The proliferation of cells within the dermis is stimulated by several factors produced by macrophages in the wound bed, so proliferation was compared in knockout and control mice. Ki-67 immunohistochemical staining was used to identify proliferating dermal cells **(Figure 10)**. Image analysis was used to determine the density of Ki-67-positive cells in the wound bed. No statistical differences were observed.

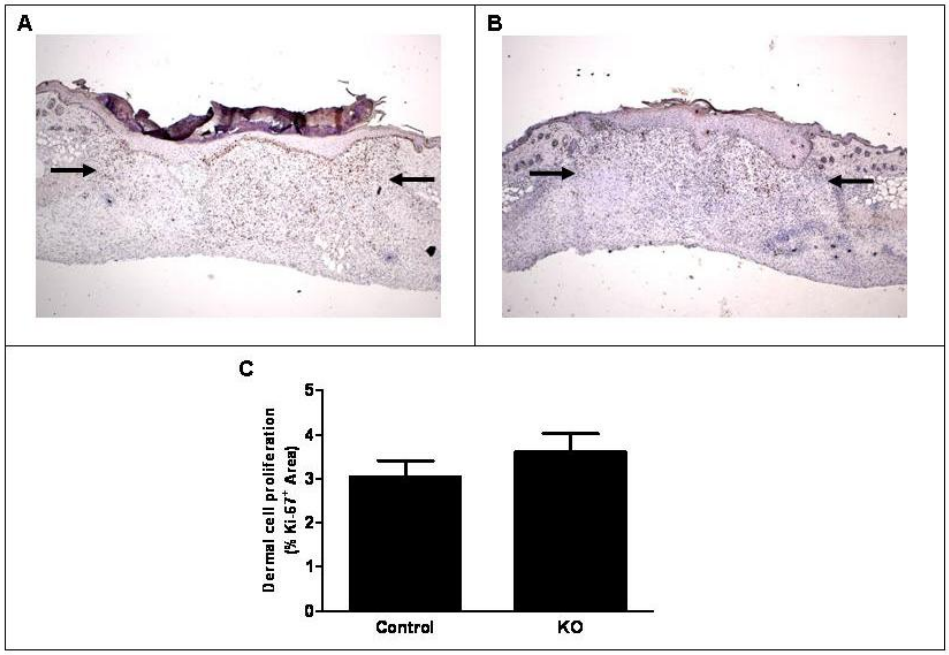

Figure 10: Dermal cell proliferation in control and knockout wounds. Immunohistochemical staining for the proliferation marker Ki-67 was used to detect proliferating cells in 5 day wounds. Representative images of wounds from a control mouse (A) and knockout mouse (B) are shown. The margins of the wound bed are marked by arrows. Stained sections were analyzed using image analysis software to determine the density of dermal cell proliferation, or the percent area of the total wound bed stained positive for Ki-67 (C). Bars represent mean blood vessel density +/- S.E.M.; p=0.3088; n=19 control wound and n=16 knockout wounds.

Chapter 4:

DISCUSSION

Wound healing occurs in three distinct yet overlapping phases: inflammation, proliferation, and scar formation (Singer, 1999; Gurtner, 2008). Macrophages, mature myeloid cells, are recruited to the wound bed during the inflammatory stage and play an important role through the healing process. They regulate wound healing in a number of ways. Macrophages initially perform phagocytic activities, engulfing bacteria and other debris (Rodero, 2010). Macrophages also stimulate the inflammatory response, and upon activation, they secrete a large number of cytokines and other pro-inflammatory mediators (Koh, 2011). During the proliferative phase, macrophages mediate angiogenesis as well as fibroblast proliferation and recruitment (Leibovich, 1975). Fibroblasts produce collagen, which forms the new matrix of the wound bed (Delavary, 2011). It is very apparent that recruitment and proper activation of macrophages is critical for proper wound healing to occur. Studies have suggested that the pro-angiogenic growth factor VEGF may stimulate macrophage migration (Barleon, 1996; Sawano, 2001); however, the exact role of VEGF in the recruitment of macrophages to the wound bed has not been characterized.

VEGF is produced in high quantities within the wound bed, both by keratinocytes and infiltrating immune cells (Brown, 1992; Nissen, 2008; Willenborg, 2012). Previously, VEGF has been established as an angiogenic factor, mediating the growth of new blood vessels. However, recent studies have shown that macrophages express VEGFR-1 and will migrate in response to VEGF *in vitro* (Barleon, 1996; Sawano, 2001). According to these findings, we anticipated that VEGF could play an important part in the recruitment of macrophages within a

wound environment *in vivo*. We decided to explore this hypothesis to enhance our understanding of the role of VEGF in wound healing.

To test this hypothesis, we created mice with macrophages that cannot respond to VEGF because they lack expression of VEGFR-1. We first showed that we were able to effectively ablate VEGFR-1 expression in macrophages in conditional knockout mice. After careful analysis of wounds collected after five days of healing, we were able to determine that fewer macrophages are recruited to the wound in knockout mice due to inhibition of the VEGF:VEGFR-1 pathway. This first step validated our model and supported our hypothesis that VEGF regulates macrophage recruitment to the wound. While the number of macrophages found within the wound was significantly decreased, there was not a complete reduction. Some of these macrophages may have been resident macrophages, macrophages that are already present before injury. Other macrophages may have been recruited by other chemotactic proteins released during initial inflammatory response, such as monocyte chemotactic protein (MCP)-1 and granulocyte-macrophage colony-stimulating factor (GM-CSF) (DiPietro, 2001; Fang, 2007).

Because macrophages play such a critical role in mediating wound healing processes, we anticipated that decreased macrophage recruitment would have detrimental effects on wound healing in knockout mice. We showed that the percentage of completely healed wounds was less in knockout mice compared to control mice. Normally, macrophages secrete growth factors and cytokines that induce growth and proliferation of keratinocytes (Rodero, 2010), enabling wound closure. Therefore, a reduction in macrophages due to impaired recruitment

could result in delayed closure. Although we did see a 27% reduction in incidence of complete wound closure in knockout mice, the mean percentage of the wound bed covered by new keratinocytes was not statistically different between the two groups. This may be attributed to high variability between samples. A number of factors could contribute to this variability. Due to small litters and difficulty breeding, we were not able to generate a large number of mice to wound at one time. Therefore the mice were wounded in groups as the mice were available. While the ages of the mice remained constant, they were wounded at different times, which could lead to the observed variability.

Because there was delayed wound closure, we anticipated decreased cellular proliferation within the wound bed. Interestingly, we did not see significantly reduced levels of proliferation within the dermis. This may suggest that the level of macrophage reduction in knockout wounds did not go below a threshold needed to see an overall reduction in dermal cell proliferation. During wound healing, the dermis primarily consists of fibroblasts, which proliferate and migrate to mediate steps of wound repair. It is also important to note that other cell types, in addition to macrophages, produce important fibroblast growth factors (Martin, 1997; Singer 1999), which may have enabled fibroblast proliferation and migration even though there were less macrophages. Perhaps other immune cells such as neutrophils and mast cells, could have contributed to fibroblast proliferation in knockout wounds. Additionally, we anticipated reduced levels of angiogenesis in knockout wounds because macrophages secrete VEGF (Brown, 1992; Nissen, 2008; Koh, 2011), which is important for wound angiogenesis. A reduction in angiogenesis in knockout wounds could help explain delayed wound healing because angiogenesis is necessary to provide nutrients and oxygen to

sustain wound healing processes. Surprisingly, analysis of angiogenesis did not yield significant differences between knockout wounds and control wounds. Examining additional time points, between 10 and 14 days when blood vessel density peaks (Martin, 1997), may be more helpful in determining the full effect of macrophage VEGFR-1 depletion on angiogenesis.

One aspect that we did not examine in this study, but might be important in explaining the role of VEGF:VEGFR-1 is macrophage subtypes. Because we only measured overall macrophage numbers, we may have missed a shift in the macrophage phenotype resulting from a disrupted VEGF:VEGFR-1 pathway. Typically, wound associated macrophages are divided into two sub-groups: M1 (classically activated) and M2 (alternatively activated) (Novak (2013). We know that the overall efficiency of wound healing depends on the proper balance of M1 and M2 macrophages. M1 macrophages are critical during the initiation of inflammation phase (Novak, 2013). M2 macrophages are important later in the wound healing process because they resolve inflammation and mediate processes such as angiogenesis and collagen deposition (Koh, 2011; Novak, 2013). Both phenotypes are important, but over-activation or reduction of one sub-type could have detrimental effects on the healing of a wound. As seen in previous studies, excessive inflammation delays wound healing (Khanna, 2010). It is possible that by inhibiting the VEGF:VEGFR-1 pathway, we are selectively reducing the M2 phenotype. If this were to occur, we would anticipate a prolonged inflammation phase, and subsequently, a delay in wound healing similar to what we observed in our studies.

Overall, we showed that VEGF is critical in the recruitment of macrophages to the wound bed during healing, because that disruption of the VEGF:VEGFR-1 pathway causes a

reduction of macrophages within the wound. We also showed that when a reduction of macrophages is observed, we see delayed wound healing. Going forward in this project, it would be important to investigate more time points to better characterize the effect of the macrophage reduction throughout the wound healing process. It would also be important to investigate the phenotypes of macrophages within the wounds of knockout and control mice to determine whether disruption of the VEGF:VEGFR-1 pathway shifts activation of either phenotype. Investigating VEGF signaling in macrophage recruitment further will be important in establishing a new role of VEGF in wound healing and expanding our understanding of abnormal healing.

REFERENCES

Barleon B, Sozzani S, Zhou D, Weich HA, Mantovani A, Marmé D. Migration of Human Monocytes in Response to Vascular Endothelial Growth Factor (VEGFR) is Mediated via the VEGF Receptor flt-1. *Blood*. 1996; 87(8): 3336-3343.

Brown LF, Yeo KT, Berse B, Yeo TK, Senger DR, Dvorak HF, van de Water L. Expression of vascular permeability factor (vascular endothelial growth factor) by epidermal keratinocytes during wound healing. *J Exp Med*. 1992; 176(5): 1375-1379.

Clark RAF. The molecular and cellular biology of wound repair, 2nd edition. *Plenum Press*. 1996; p 4.

Clausen BE, Burkhardt C, Reith W, Renkawitz R, Förster I. Conditional gene targeting in macrophages and granulocytes using LysMcre mice. *Transgenic Research*. 1999; 8:265-277.

Devalary BM, van der Veer WM, van Egmond M, Niessen FB, Beelen RH. Macrophages in Skin Injury and Repair. *Immunobiology* 2011; 216: 753-762.

DiPietro LA, Reintjes MG, Low QE, Levi B, Gamelli RL. Modulation of macrophage recruitment into wounds by monocyte chemoattractant protein-1. *Wound Repair Regen*. 2001; 9(1): 28-33.

Dvorak HF. Angiogenesis: update 2005. *J Thromb Haemost*. 2005; 3: 1835-1842.

Fang Y, Gong SJ, Xu YH, Hambly BD, Bao S. Impaired cutaneous wound healing in granulocyte/macrophage colony-stimulating factor knockout mice. *Br J Dermatol*. 2007; 157(3): 458-465.

Ferrara N, Gerber HP, LeCouter J. The biology of VEGF and its receptors. *Nat Med.* 2003; 9(6): 669-679.

Gurtner GC, Wemer S, Barradon Y, Longaker MT. Wound repair and regeneration. *Nature*. 2008; 453(7193): 314-321.

Khanna S, Biswas S, Shang Y, Collard E, Azad A, Kauh C, Bhasker V, Gordillo GM, Sen CK, Roy S. Macrophage dysfunction impairs resolution of inflammation in the wounds of diabetic mice. *PloS One*. 2010; 5(3).

Koh TJ, DiPietro LA. Inflammation and wound healing: the role of the macrophage. *Expert Reviews in Molecular Medicine.* 2011; 13(23): 1-12.

Leibovich SJ, Ross R. The role of the macrophage in wound repair. A study with hydrocortisone and antimacrophage serum. *Am J Pathol*. 1975; 78(1): 71-100.

Lucas T, Waisman A, Ranjan R, Roes J, Krieg T, Müller W, Roers A, Eming SA. Differential roles of macrophages in diverse phases of skin repair. *Journal of Immunology*. 2010; 184: 3964-3977.

Man XY, Yang XH, Cai SQ, Yao YG, Zheng M. Immunolocalization and expression of vascular endothelial growth factor receptors (VEGFRs) and neuropilins (NRPs) on keratinocytes in human epidermis. *Mol Med*. 2006; 12(7-8): 127-136.

Martin P. Wound healing – aiming for perfect skin regeneration. *Science*. 1997; 276: 75-81.

Mizra R, DiPietro LA, Koh TJ. Selective and specific macrophage ablation is detrimental to wound healing in mice. *Am J Pathol*. 2009; 175(6): 2454-2462.

Nissen NN, Polverini PJ, Koch AE, Volin MV, Gamelli RL, DiPietro LA. Vascular endothelial growth factor mediates angiogenic activity during the proliferative phase of wound healing. *Am J Pathol*. 1998; 152(6): 1445-1452.

Novak ML, Koh TJ. Macrophage phenotypes during tissue repair. *J Leukoc Biol*. 2013

Rodero MP, Khosrotehrani K. Skin wound healing modulation by macrophages. *Int J Clin Exp Pathol*. 2010; 3(7): 643-653.

Sawano A, Iwai S, Sakurai Y, Ito M, Shitara K, Nakahata T, Shibuya M. Flt-1, vascular endothelial growth factor receptor 1, is a novel cell surface marker for the lineage of monocyte-macrophages in humans. *Blood*. 2001; 97(3): 785-791.

Singer AJ, Clark RA. Cutaneous wound healing. *N Engl J Med.* 1999; 341: 738-749.

Wilgus TA, Matthies AM, Radek KA, Dovi JV, Shankar R, DiPietro LA. Novel function for vascular endothelial growth factor receptor -1 on epidermal keratinocytes. *Am J Pathol*. 2005; 167(5): 1257-1266.

Willenborg S, Lucas T, van Loo G, Knipper JA, Krieg T, Haase I, Brachvogel B, Hammerschmidt M, Nagy A, Ferrara N, Pasparakis M, Eming SA. CCR2 recruits an inflammatory macrophage supopulation critical for angiogenesis in tissue repair. *Blood*. 2012; 120(3): 613-625.